NOTE

Effects of application rate and cold soaking pretreatment of *Pisolithus* spores on effectiveness as nursery inoculum on western conifers[1]

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Received September 28, 1982[2]
Accepted February 7, 1983

ALVAREZ, I. F., and J. M. TRAPPE. 1983. Effects of application rate and cold soaking pretreatment of *Pisolithus* spores on effectiveness as nursery inoculum on western conifers. Can. J. For. Res. **13**: 533–537.

Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.), Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), Shasta red (*Abies magnifica* var. *shastensis* Lemm.), and white fir (*Abies concolor* (Gord. & Glend.) Lindl. ex Hild.) seedlings were inoculated in a bare root nursery with basidiospores of *Pisolithus tinctorius* (Pers.) Coker & Couch. The spores were applied at three rates with or without cold–wet pretreatment of 7 or 21 days. Pretreatment did not affect spore efficiency as inoculum. Only ponderosa pine responded to inoculation. Inoculations in the greenhouse with a wider range of spore application rates revealed that a higher concentration of spores was needed to induce an increase in growth and mycorrhiza formation of Douglas-fir than ponderosa pine. These levels were much higher than those used in nursery inoculations.

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Dans une pépinière pour la production de plants à racines nues, on a inoculé des semis de *Pinus ponderosa* Dougl. ex Laws., de *Pseudotsuga menziesii* (Mirb.) Franco, d'*Abies magnifica* var. *shastensis* Lemm. et d'*Abies concolor* (Gord. & Glend.) Lindl. ex Hild., avec des basidiospores de *Pisolithus tinctorius* (Pers.) Coker & Couch. Les spores furent appliquées à trois concentrations avec ou sans prétraitement de mouillage à froid de 7 ou 21 jours. Le prétraitement n'a pas affecté l'efficacité des spores comme inoculum. Seul le *P. ponderosa* a réagi à l'inoculation. Des inoculations exécutées en serres avec une gamme plus étendue dans les applications de spores, ont montré que pour provoquer une croissance accrue et une formation de mycorhizes supérieures chez le *P. menziesii* par rapport au *P. ponderosa*, il fallait une plus forte concentration des spores. Ces concentrations étaient en outre plus fortes que celles utilisées dans les inoculations en pépinière.

[Traduit par le journal]

Introduction

Outplanted tree seedlings with *Pisolithus tinctorius* (Pers.) Coker & Couch mycorrhizae survive and grow better on many sites than seedlings without *P. tinctorius* ectomycorrhizae (Marx *et al.* 1977; Baer and Otta 1981; Dixon *et al.* 1981; Ruehle *et al.* 1981). At least half of the root system needs to be colonized by *P. tinctorius* to achieve this enhancing effect of southern pine seedlings (Marx *et al.* 1977; Kais *et al.* 1981). Mycelial inoculum has proven more conducive to mycorrhiza formation than spore inoculum in the studies reported so far (Marx *et al.* 1976).

Millions of seedlings are outplanted each year in the Pacific Coast states. Successful methods for inoculation of either bare root or containerized stock with vegetative inoculum of *P. tinctorius* have yet to be developed; several attempts thus far have failed. Moreover, no one fungal ecotype is likely to be adaptable to all of the great diversity of sites in mountainous terrain. *Pisolithus tinctorius* spores, however, are readily available. Large sporocarps, some yielding up to 200 g of spores, occur abundantly from early summer to fall in many localities. Spores in the cool, moist climate of the Pacific Northwest may need exposure to cold, wet conditions to overcome dormancy much as seeds of conifers in the region need stratification for prompt and uniform germination. We undertook this study to determine optimum rate of spore application needed to achieve acceptable levels of *P. tinctorius* mycorrhizae and to test whether exposure of the spores to cold, wet conditions would alter their potential as inoculum and increase mycorrhiza formation with seedlings in two bare root nurseries.

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[1] Oregon Agricultural Experiment Station Technical Paper No. 6539.

[2] Revised manuscript received February 3, 1983.

Materials and methods

Spore collection and storage

Pisolithus tinctorius sporocarps were collected in the Siskiyou Mountains, northern California, in fall 1979 from under ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.), Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), white fir (*Abies concolor* (Gord. & Glend.) Lindl. ex Hild.), and Shasta red fir (*Abies magnifica* var. *shastensis* Lemm.) at elevations from 900 to 1500 m. Spores were extracted by vacuum and labeled according to host species and elevation at which the sporocarps had been collected. They were stored in the dark at 5°C (Marx 1976) until used. A mixture by host species and elevation was prepared for use in the nursery and greenhouse inoculations. One milligram of mixture contained 1.2×10^6 spores.

Nursery inoculations

The two bare root nurseries chosen for the study were Tyee at Umpqua, Oregon, and H.H. at Sebastopol, California. Tyee was inoculated in late May, at time of sowing. Nursery H.H. was inoculated in early June in beds previously sown in late April. Conventional nursery practices were followed in both nurseries throughout the study. The experiments in both nurseries were in a randomized block design with four replications per treatment.

In Tyee, 10 treatments were tested on all four tree species: three spore concentrations: 0.05, 0.1, and 0.2 g of spores/ft^2 (1 ft = 0.3048 m); three durations of soaking of spores at 1.6°C: 0, 7, and 21 days; and control, no spores applied. The experimental unit was a plot 4×4 ft. Sown buffer plots of the same size were left between treatments.

In H.H., three treatments were tested on ponderosa pine only: 0.1 g of spores/ft^2 without and with a 21-day cold soak, and control. The experimental unit was a plot 3×3 ft. Sown buffer plots of the same size were left between treatments.

The spores were weighed to achieve the required concentration per surface area for each plot and suspended in water with a small amount of Tween-20 surfactant. Cold soaking treatments were begun 21 and 7 days, respectively, prior to inoculation. Treatments with no cold exposure were prepared the day of inoculation. All spore suspensions were transported to the nursery in an ice chest. In the nursery, spore allotments for each plot were suspended in 5 L of water and applied evenly over the plot surface with a watering can.

Seedlings were carefully dug out of the plots at the end of the first growing season in October at Tyee, and November at H.H. Fifty seedlings were randomly selected from the total dug from each plot, wrapped in wet paper towels, and transported in plastic bags to the laboratory in ice chests. Within 2 weeks or less of harvest, seedlings were examined to remove any with damaged roots. Ten of the remaining seedlings were then randomly chosen and measured for shoot height, stem diameter, and percent of feeder rootlets mycorrhizal with *P. tinctorius* and other fungi. The seedlings were then oven-dried and tops and roots weighed.

Analysis of variance was performed to test for differences among rates of application, length of cold treatment, their interactions, and control versus factorial for each variable within each species. For variables that differed significantly by treatment, means were compared by Tukey's test ($P \leq 0.05$).

Greenhouse inoculations

Soil from Tyee nursery was mixed with coarse vermiculite 1 : 1 (v/v) and steam pasteurized (70°C/30 min). Forty-eight pots 14 cm in diameter and 12 cm deep were filled with this mixture. Twenty-four were each sown with 8 stratified seeds of Douglas-fir, and 24 with 10 seeds of ponderosa pine. Seeds started to germinate 2 weeks after sowing. Four weeks after sowing, seedlings were thinned to four per pot, the height of each seedling recorded and inoculation treatments applied.

The treatments consisted of 0.001, 0.01, 0.1, 1, 10 g of spores per pot, and control. Control treatments received no spores because Marx (1976) found that killed spores did not affect seedling response. The spores were mixed with 40 g of the growing medium and applied as a surface layer. Steam-sterilized, fine quartz gravel was applied as an additional layer to prevent splashing and drying of the inoculum layer. After inoculation each pot was mist-sprayed to infiltrate spores into remaining soil volume. The pots were then placed randomly on a greenhouse bench where they remained throughout the experiment. Fertilization was limited to one application of 20−19−18 N−P−K fertilizer at the rate of 6.1 g/m^2 midway through the experiment. Manual watering as needed was done carefully to prevent splashing. The seedlings were harvested 6 months after inoculation.

Total numbers of short roots were counted as well as estimated from measurements of a subsample of six primary laterals sampled in a stratified manner. Dry weights of all primary laterals were recorded for each seedling, and total number of short roots calculated as number short roots in subsample × dry weight of all primary laterals per dry weight of primary laterals in subsample. Percentage of *P. tinctorius* mycorrhizae was estimated visually for the complete root system as well as calculated from counts of the subsample.

The statistical design was completely randomized. Each treatment was replicated four times with four observations per replication. Each tree species was analyzed separately. Means of variables found to differ significantly in the analysis of variance were compared by Tukey's test ($P \leq 0.05$).

Results

Nursery inoculations

For all species in both nurseries, length of cold soaking of spores produced no significant effects. Only ponderosa pine responded significantly to spore application rate in terms of growth, with the largest top and total weight occurring at the 0.05-g/ft^2 rate (Table 1). With all inoculation treatments combined, inoculated pines averaged significantly greater in dry weight of tops, roots, and tops + roots than noninoculated pines. For Douglas-fir and Shasta red fir, only stem diameter differed significantly among spore application rates. No values for application rates combined differed significantly from controls (Table 1). Growth of white fir was

TABLE 1. Growth and ectomycorrhiza development of 5-month-old Tyee nursery seedlings inoculated with *Pisolithus tinctorius* (*Pt*) spores. Values for inoculation treatments are the means of 120 seedlings; controls had 40 seedlings

Species	Application rate (g/ft^2)	Spores/cm^2 soil surface × 10^4	Height (cm)	Stem diameter (mm)	Dry weight (g)			Mycorrhizae (%)		
					Top	Root	Total	*Pt*	Other	Total
Pinus ponderosa	0.05	6.5	5.5*a*	2.1*a*	0.35*a*	0.17*a*	0.52*a*	6*a*	40*a*	46*a*
	0.1	13.0	5.4*a*	2.1*a*	0.34*ab*	0.16*a*	0.50*ab*	3*a*	41*a*	44*a*
	0.2	26.0	5.0*a*	2.0*a*	0.29*b*	0.15*a*	0.44*b*	4*a*	35*a*	39*a*
	0	0	5.0	2.0	0.27*	0.13*	0.40*	0	33	33
Pseudotsuga menziesii	0.05	6.5	5.2*a*	1.4*ab*	0.20*a*	0.14*a*	0.34*a*	0*a*	54*a*	54*a*
	0.1	13.0	5.6*a*	1.5*a*	0.20*a*	0.13*a*	0.33*a*	1*a*	52*a*	53*a*
	0.2	26.0	5.0*a*	1.3*b*	0.16*a*	0.12*a*	0.28*a*	0*a*	57*a*	57*a*
	0	0	5.4	1.4	0.19	0.14	0.33	0	49	49
Abies magnifica var. *shastensis*	0.05	6.5	5.1*a*	1.5*a*	0.24*a*	0.11*a*	0.35*a*	1*a*	6*a*	7*a*
	0.1	13.0	5.3*a*	1.4*b*	0.23*a*	0.11*a*	0.34*a*	0*a*	11*a*	11*a*
	0.2	26.0	5.4*a*	1.4*b*	0.21*a*	0.10*a*	0.31*a*	0*a*	7*a*	7*a*
	0	0	5.3	1.4	0.20	0.11	0.31	0	12	12
Abies concolor	0.05	6.5	3.7*a*	1.6*a*	0.19*a*	0.10*a*	0.29*a*	0*a*	22*a*	22*a*
	0.1	13.0	4.2*a*	1.7*a*	0.20*a*	0.11*a*	0.31*a*	1*a*	38*b*	39*a*
	0.2	26.0	4.3*a*	1.6*a*	0.20*a*	0.12*a*	0.32*a*	0*a*	36*b*	36*a*
	0	0	4.2	1.7	0.20	0.12	0.32	0	32	32

NOTE: Means in a column, within a tree species sharing a common letter do not differ significantly by Tukey's test at $P \leq 0.05$.
*Denotes significant differences between inoculated and control treatments based on *F*-test ($P \leq 0.05$).

TABLE 2. Growth and ectomycorrhiza development of 6-month-old H.H. nursery ponderosa pine seedlings inoculated with *Pisolithus tinctorius* (*Pt*) spores. Values for inoculation treatments are the means of 80 seedlings; controls had 40 seedlings

Application rate (g/ft^2)	Spores/cm^2 soil surface × 10^4	Height (cm)	Stem diameter (mm)	Dry weight (g)			Mycorrhizae (%)		
				Top	Root	Total	*Pt*	Other	Total
0.1	13.0	11.6	3.3	1.62	0.82	2.44	15	85	100
0	0	11.9	3.4	1.76	0.87	2.63	1*	96	97

*Denotes significant differences between inoculated and control treatments based on *F*-test ($P \leq 0.05$).

unaffected by inoculation.

Ponderosa pine growth in H.H. was not affected by inoculation. However, the percentage of *P. tinctorius* mycorrhizae was significantly larger in the inoculated treatment than in the control and more than twice as high as the percentage obtained in Tyee (Table 2).

The levels of *P. tinctorius* mycorrhizae attained were low for all species and concentrations, well below the minimum levels reported to be needed to increase field survival and growth of pine seedling when outplanted in the southeastern United States (Marx *et al.* 1977; Kais *et al.* 1981; Ruehle *et al.* 1981). Seedlings from Tyee nursery had 7–57% mycorrhizal colonization of short roots, mostly with *Thelephora* sp. (Table 1), and those at H.H. had 97–100% colonization, mostly with fungi other than *P. tinctorius* (Table 2).

Greenhouse inoculations

The calculated total numbers of short roots were significantly correlated with the actual total numbers of short roots ($r = 0.95$ for Douglas-fir and 0.93 for ponderosa pine, standard errors of 35 for Douglas-fir and 74 for ponderosa pine). Correlation analysis of percentage *P. tinctorius* mycorrhizae measured visually with percentage calculated gave $r = 0.96$ and 0.95 for Douglas-fir and ponderosa pine, respectively, with a standard error of 5 for both species. Analysis of variance for total number of short roots and percentage of *P. tinctorius* mycorrhizae measured by the two methods gave similar results.

Growth of Douglas-fir measured as fresh weight of the top and height increase from time of inoculation to harvest was significantly increased by inoculation with 10 g of spores per pot over all other treatments, which did not differ significantly from each other. Two seedlings from the 10-g treatment showed deep blue coloration in the meristematic region of their primary roots. The unknown cause seemed physiological rather than microbial. Significantly greatest colonization of roots by *P. tinctorius* occurred in treatments with 1 g of spores, with percentage of *P. tinctorius* mycorrhizae ranging from 22 to 75 and averaging 45.

The results for ponderosa pine are difficult to interpret due to unexplainable *P. tinctorius* contamination of controls. The trend seems to indicate that lower application rates are needed for mycorrhiza formation in ponderosa pine than in Douglas-fir.

Discussion

Large-scale tests of vegetative *P. tinctorius* inoculum in western bare root nurseries have been unsuccessful so far (D. Marx and R. Molina, unpublished data); similar failure of our spore inoculations, though disappointing, is thus not surprising. The reasons for the failures are unknown, although western nurseries customarily fumigate in autumn, in contrast to southern nurseries, which fumigate in spring. The southern nurseries have enjoyed considerable success with *P. tinctorius* inoculation in the same series of tests (D. Marx, personal communication).

Douglas-fir seedlings inoculated with 10 g of *P. tinctorius* spores per pot in the greenhouse had a mean top growth 1.5 times larger than control seedlings. Increased top growth was not correlated with increased root dry weight, number of short roots, or higher colonization by the fungus: highest percentages of mycorrhizae were obtained with the application of 1 g of spores per pot. The high rate of spore application needed to affect Douglas-fir growth or mycorrhiza development in the greenhouse suggests that application rates in the nursery were too low for Douglas-fir, Shasta red fir, and white fir seedlings.

Mycorrhiza formation in ponderosa pine seemed to require lower rates of spore application than Douglas-fir. Percentages of mycorrhizae were higher for ponderosa pine than for Douglas-fir for the same rate of spore application up to a threshold value. Above the threshold, mycorrhiza formation in both species appeared to correlate negatively with increasing rates of spore application. Self-inhibition may be present at high spore concentrations, as suggested by Marx (1976). Ruehle (1980), using an application rate of 7.5×10^4 spores/cm^2, found that regardless of seedling age at time of inoculum, levels of *P. tinctorius* mycorrhizae would not exceed 10%. Our results are similar. Germinability of *P. tinctorius* spores can be low (Lamb and Richards 1974) and probably variable, depending on method of spore extraction and conditions and time in storage. At least 20% of *P. tinctorius* spores may be aberrant (Mims 1980).

Cold soaking of spores for up to 21 days did not affect mycorrhiza formation in our experiments. Given the hypothesis that overwintering of spores in the field takes place prior to germination, however, longer cold treatment combined with leaching still merits exploration. Additional research is also needed on the overall ecology and physiology of *P. tinctorius* spores to increase their efficiency as a source of inoculum for mycorrhiza formation.

Acknowledgements

We thank the management of Tyee and H.H. nurseries and Mr. J. N. Freemyers, Fruit Growers Supply Co., for their cooperation. These studies were funded in part by the Boise-Cascade Corp., Champion International Corp., Fruit Growers Supply Co., International Paper Co., Medford Corp., Roseburg Lumber Co., Southern Pacific Land Co., Sun Studs Corp., and the Bureau of Land Management.

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